# METABONOMICS OF PIG BLOOD PLASMA FOLLOWING WHOLE BODY EXPOSURE TO LOW LEVELS OF GB VAPOR

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Chemometric approaches have the potential to enhance defense operations against chemical warfare agents (CWAs), for which complex data must be analyzed. Therefore, we have begun an exploration of the use of chemometrics for the correlation of metabolic change with low-level CWA exposure in animal models. In this exploratory study, pigs were exposed to GB (sarin) over a range of concentrations (0.028 – 0.310 mg/cubic meters) and exposure times (0-180 minutes). Blood (4-8 mL) was collected periodically during miosis-level exposure and the plasma analyzed by proton nuclear magnetic resonance spectroscopy (NMR) at 500 MHz. Principle component (PC) analysis of a small preliminary data set resulted in a PC2, PC1 scores plot showing clustering of the spectra from exposed pigs. Variance highlighted by the first and second principal components corresponded to specific spectral regions, which were tentatively assigned to EDTA /choline (NMR peaks overlap) and lactate. Lactate concentration was found to vary in a parallel clinical analysis. The results of this initial study indicate that a combination of chemometrics and NMR will yield metabonomics data useful for establishing biochemical markers for agent exposure. Ideally, such markers would identify metabolic changes that occur prior to visible external symptoms (e.g. miosis, convulsions).

## INTRODUCTION

The development of adequate troop protection against chemical warfare agents (CWAs) requires an understanding of the effect of low exposure levels of CWAs. Traditional methods for the study of low-level exposure to nerve agents involve animal models (pig, rat, etc.) and have included determination of time to miosis and the quantitation of bound agent in the blood by regeneration assays.[1,2] Of concern are metabolic affects that may occur prior to external signs and/or prior to the presence of detectable levels of bound agent. The analysis of metabolic affects requires methods to determine biochemical markers associated with environmental change, a field recently referred to as "metabonomics." High field nuclear magnetic resonance (NMR) has emerged as a method of choice for metabonomics of biological fluids.[3-6] We have, therefore, begun an exploration of the use of NMR and chemometrics to search for possible biochemical markers associated with low-level exposure to CWAs. Here we present initial results on the

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analysis of minipig blood plasma by high field NMR after low-level exposure to GB by whole body inhalation.

## EXPERIMENTAL METHODS

### *1. SARIN EXPOSURE & BLOOD COLLECTION:*

Male Gottingen minipigs were exposed to low levels of GB by whole body inhalation in an isolated chamber as described.[1] Pigs were treated intravenously with lactated Ringers solution to prevent dehydration. The GB exposure level was from 0.020 $mg/m^3$ to 0.310 $mg/m^3$ and the total exposure time ranged from 10 to 180 minutes. Miosis level was recorded as a function of time. Blood was collected in 4-8 mL volumes periodically during miosis level exposure into BD Vacutainer tubes containing $K_3$EDTA as an anticoagulant (Fisher Scientific, Pittsburgh, PA). Whole blood was centrifuged[2] and the plasma stored at $-20^{O}$C. Vials containing plasma were thawed on ice, mixed with a vortexer, and ~300 microliter aliquots transferred to microfuge tubes for NMR sample preparation.

### *2. NMR SAMPLE PREPARATION:*

For each sample, 350-434 mL of $D_2O$, containing 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) as a chemical shift reference, was added to 250-310 mL of plasma. All NMR samples contained the same ratio of $D_2O$ volume relative to plasma volume. The mixture was centrifuged for 20 minutes at $4^{O}$C and 3710 *g* (Beckman Coulter Microfuge® 22R, F241.5 rotor) and the supernatant was transferred to a 5 mm NMR tube. $D_2O$ and DSS were purchased from Sigma-Aldrich, St. Louis, MO.

### *3. NMR EXPERIMENTS:*

$^1$H 500 MHz 1D NMR experiments were carried out on a Bruker Avance DMX spectrometer using an inverse detection broadband probe. Experiments included one or more of the following: 1D pre-saturation nuclear Overhauser effect (NOESY)[7], Carr-Purcell-Meiboom-Gill[7], or water suppression using excitation sculpting with gradients and a squiggly pulse.[8,9] Typical parameters used were 64 scans, 4 dummy scans, and a 1-2 second relaxation delay.

### *4. CHEMOMETRICS:*

Principal component analysis was carried out with one or more of the following software packages: AMIX[10], Partek[11], and/or The Unscrambler.[12] The water chemical shift region was excluded from calculations. Calculations were explored in which other regions were also excluded. To date, calculations have included small sample sets of ~10 spectra with all spectra in a particular calculation collected with the same NMR pulse program.

## RESULTS & DISCUSSION

### *1. ASSIGNMENT OF PIG PLASMA NMR SPECTRUM:*

Most of the peaks in the NMR spectrum of pig plasma were identified by comparison with human or rat plasma spectra in the literature.[3-6] Peaks corresponding to free, $Ca^{2+}$-complexed and $Mg^{2+}$-complexed EDTA (CaEDTA and MgEDTA, respectively) were identified by spiking samples with stock solutions of EDTA, $CaCl_2$ and $MgCl_2$. Figure 1 shows spectra from various exposure times for a representative minipig. Assignments are indicated in Figure 2.

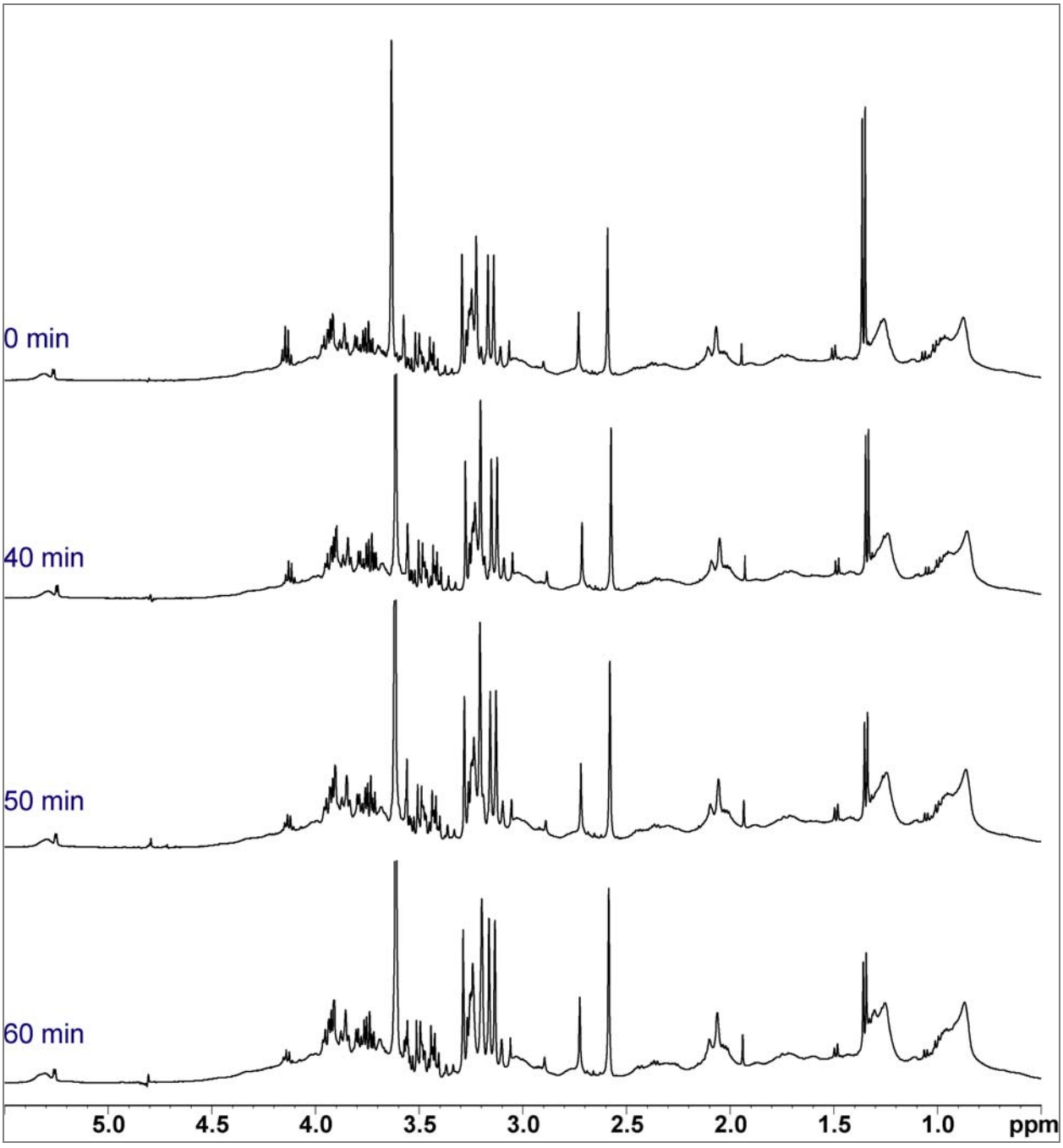



***FIGURE 1:*** Spectra for a representative minipig. Total exposure time = 60 minutes, 0.044 mg/m$^3$. Time to maximum miosis (21%) = 76 minutes.

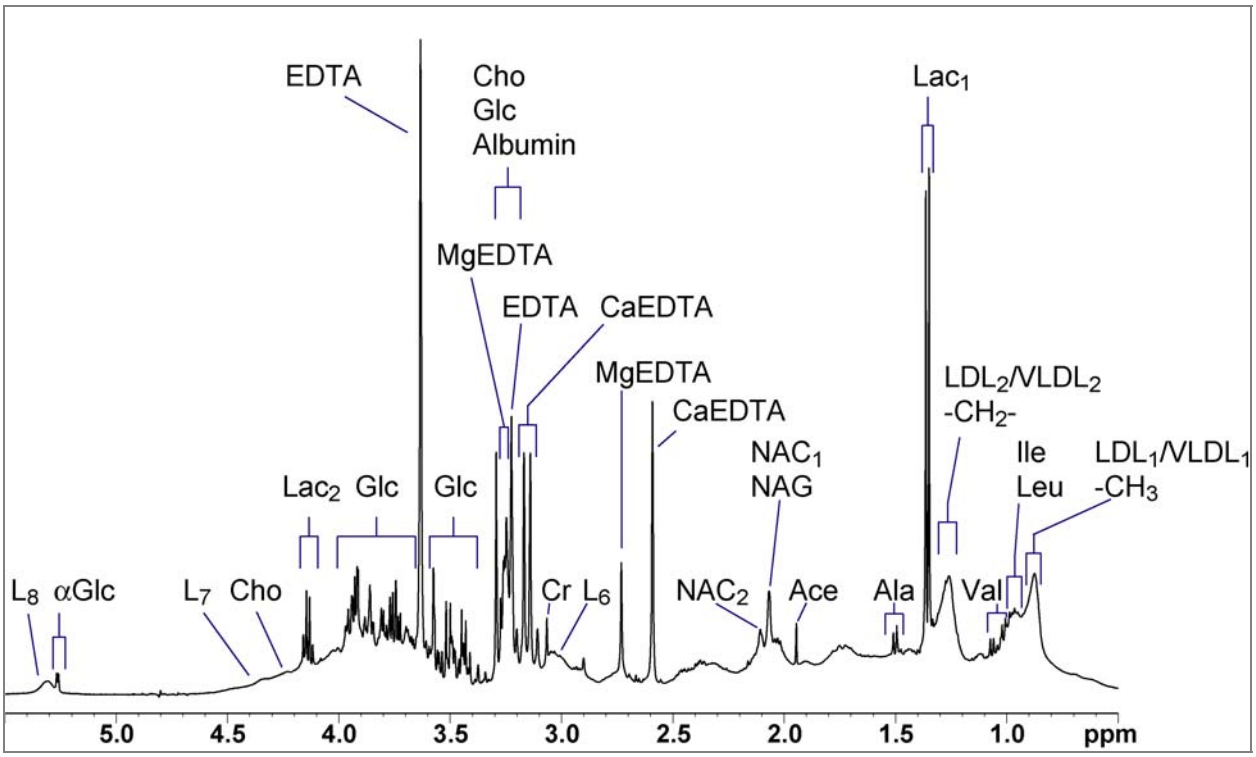
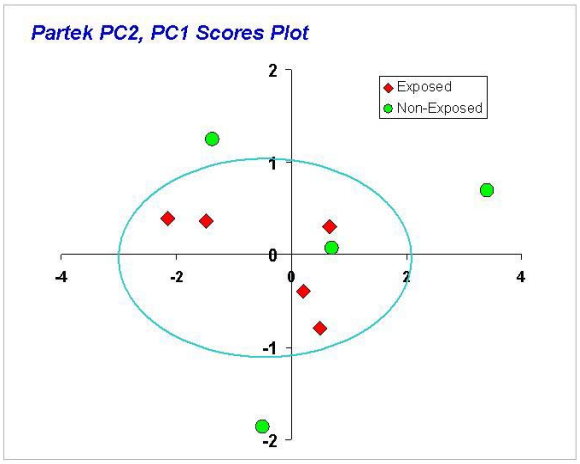
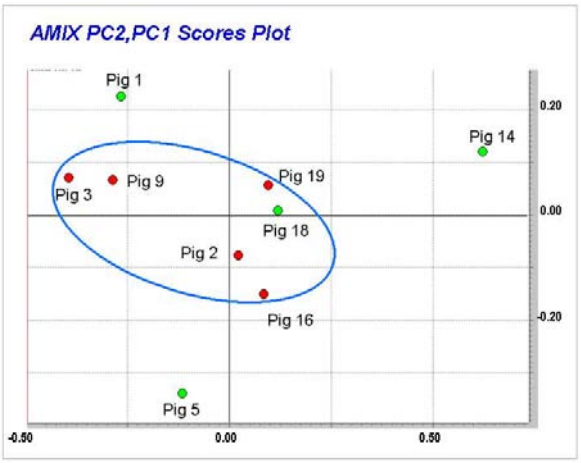



***FIGURE 2:*** Control spectrum of minipig plasma at exposure time = 0 minutes. Assignment abbreviations: glucose (Glc), choline-containing compound (Cho), acetate (Ace), alanine (Ala), lactate (Lac), lipid (L), N-acetyl groups (NAc, NAG), isoleucine (Ile), leucine (Leu), valine (Val).

## *2. PRINCIPAL COMPONENT ANALYSIS:*

Preliminary analysis has been carried out using different software packages. The PC1, PC2 Scores plots below show some clustering of spectra for exposed pigs.



As shown in Figure 3, the loadings plots indicate that the primary variation between spectra results from intensity differences that occur in the following spectral regions (ppm): 1.2-1.3, 1.3-1.4, 3.1-3.2, 3.2-3.3, 3.6-3.7. These regions correspond to EDTA (free and complexed), lactate, LDL/VLDL, and choline-containing compounds. However, the current PCA results must be taken with caution. The variation in free EDTA concentration is expected to result inadvertently from the method of sample collection. Though the amount of EDTA in each vacutainer may be constant, the exact volume of blood collected varies between samples. On the other hand, the concentration of complexed EDTA would depend on the concentrations of $Ca^{2+}$ and $Mg^{2+}$ ions in the plasma, provided that an excess of EDTA is present (as is the case here). The $Ca^{2+}$ and $Mg^{2+}$ concentrations, and consequently complexed EDTA concentration, may therefore correlate with exposure. For lactate, two possible sources of concentration differences between spectra (other than exposure to agent) are the introduction of lactate via the IV lactated Ringers solution and a build up or decline of lactate in conjunction with periods of movement or rest by an individual pig. The main peaks from choline-containing compounds overlap EDTA peaks in the 3.10-3.40 ppm region. Variation due to choline has therefore not yet been established for certain.

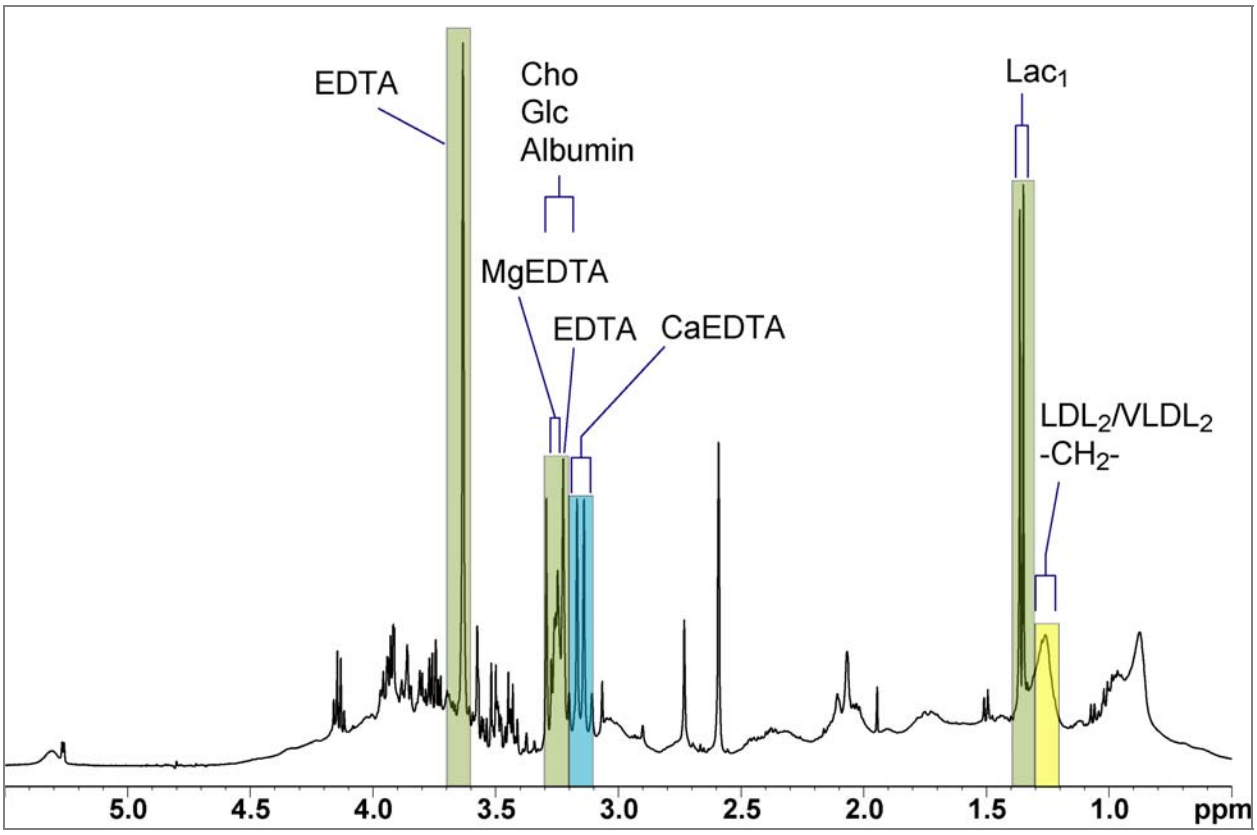



***FIGURE 3:*** PCA spectral loadings regions. Green = regions of variability identified by both Partek & AMIX. Yellow = region identified by AMIX. Blue = region identified by Partek.

### *3. FUTURE STUDIES:*

While it is possible that the concentrations of one or more of $Ca^{2+}$, $Mg^{2+}$, lactate and choline correlate with exposure, additional studies are required to verify whether such a correlation exists. We are currently continuing our efforts by: 1) carrying out NMR experiments on plasma from additional pigs to include in PCA, 2) subtracting the EDTA peak areas in the region of overlap with choline to determine

whether choline concentration varies, 3) carrying out PCA in which the lactate and EDTA peak regions are excluded from the calculations to determine if small but significant variations have been obscured by the EDTA and lactate contributions.

## CONCLUSIONS

Metabolites and other biochemical compounds and complexes are readily observable in minipig plasma by NMR spectroscopy with minimal sample preparation. Peaks have been assigned to 15 compounds or complexes. Initial PCA results are promising and indicate several spectral regions that may correlate with exposure. Whether such correlation exists must be verified by additional studies.

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**10.** AMIX 3.0, Bruker BioSpin Corporation, Billerica, MA.

**11.** Partek Pro 5.1, Partek, St. Louis, Missouri.

**12.** The Unscrambler 8.0 (© 1986-2003), Camo Process As. Nedre Vollgate 8, N-0158, Oslo, Norway.